Use of Vector Diagnostics During Military Deployments: Recent Experience in Iraq and Afghanistan

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ABSTRACT Vector-borne diseases such as malaria, dengue, and leishmaniasis are a threat to military forces deployed outside of the United States. The availability of specific information on the vector-borne disease threat (e.g., presence or absence of a specific disease agent, temporal and geographic distribution of competent vectors, and vector infection rates) allows for effective implementation of appropriate measures to protect our deployed military forces. Vector diagnostics can provide critical, real-time information crucial to establishing effective vector prevention/control programs. In this article we provide an overview of current vector diagnostic capabilities, evaluate the use of vector diagnostics in Operation Enduring Freedom and Operation Iraqi Freedom, and discuss the concept of operations under which vector diagnostics are employed.

INTRODUCTION

Military Threat of Arthropod-Borne Diseases

Arthropod-borne diseases have historically posed a significant threat to deployed military forces.[1–11] The pathogens causing these diseases are transmitted by a variety of biting arthropods, to include mosquitoes, ticks, chiggers, sand flies, lice, fleas, and biting midges. Arthropod-borne diseases considered a significant threat to military forces include malaria,[8] dengue,[6,11,12] leishmaniasis,[4,7,9,10] scrub typhus,[1,2,5] epidemic and endemic typhus,[1,5] Crimean-Congo hemorrhagic fever (CCHF) virus, Rift Valley fever (RVF) virus, Sindbis virus, sandfly fever viruses (SFV), Venezuelan equine encephalitis (VEE) virus, tick-borne encephalitis (TBE) viruses, West Nile (WN) virus and Japanese encephalitis (JE) virus. A variety of additional arthropod-borne diseases could potentially impact military operations. A summary of some of the most significant arthropod-borne threat agents,[3] to include pathogen, primary reservoir(s), and vectors, is presented in Table I.

Although vaccines and/or prophylactic drugs are the preferred method of protecting deployed military personnel from infectious diseases,[13,14] these protective measures are not available for many arthropod-borne diseases (Table I). Currently, FDA-licensed vaccines for widespread use are available for yellow fever virus, JE virus, and plague, while limited-use vaccines (normally restricted to individuals at high risk of infection) are available for VEE, RVF, CCHF, TBE, and eastern and western equine encephalitis (EEE and WEE) viruses. Prophylactic drugs are widely used for the prevention of malaria and less frequently for protection from scrub typhus and various rickettsial diseases, but provide no protection against the majority of arthropod-borne diseases (Table I).

Requirement for Vector Diagnostics

In the absence of a vaccine or prophylactic drug, the most effective means of protecting deployed military personnel from arthropod-borne diseases is to prevent infected arthropods from biting them. Prevention of bites from infected arthropods can be achieved through effective use of personal protective measures (PPMs) or by reducing vector populations. Effective PPMs include application of DEET-containing insect repellents to exposed skin, wearing a permethrin-treated uniform, and sleeping under an insecticide-treated bed net,[7,15–19] while vector populations may be reduced by judiciously using insecticides or eliminating vector habitat.[3,20,21] A key tenet of military vector control operations is that the goal is not to merely decrease vector populations but to actually

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14. ABSTRACT

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TABLE I. Twenty-Four Arthropod-Borne Diseases That Pose a Significant Threat to Deployed Military Forces[3]

Disease	1° Pathogen(s)	1° Vector(s)	1° Reservoir(s)	Vaccine	Drug	Vector Assays	
						Hand-Held Assay	Real-Time PCR Assay
Malaria	*Plasmodium falciparum/vivax*	*Anopheles* spp.	Humans	No	Yes	Yes	Yes
Dengue	Dengue virus (serotypes 1–4)	*Aedes aegypti/albopictus*	Humans	No	No	No[b]	Yes
Rift Valley fever	Rift Valley fever virus	Mosquitoes	Vertebrates	Yes[a]	No	No[b]	Yes
Chikungunya	Chikungunya virus	*Aedes aegypti/albopictus*	Humans	Yes[a]	No	No	Yes[c]
CCHF	CCHF virus	*Hyalomma* spp.	Small mammals	Yes[a]	No	No	Yes[c]
Sand fly fever	Sand fly fever viruses	*Phlebotomus* spp.	Humans	No	No	No[b]	Yes[c]
Onyong-nyong	Onyong-nyong virus	*Anopheles funestus*	Unknown	No	No	No	No
Sindbis	Sindbis virus	Mosquitoes	Birds	No	No	No	Yes[c]
Scrub typhus	*Orientia tsutsugamushi*	*Leptotromidium* spp.	Rodents	No	Yes	No[b]	Yes
Visceral leishmaniasis	*Leishmania donovani/infantum*	Phlebotomine sand flies	Humans/canids	No	No	No[b]	Yes
Epidemic typhus	*Rickettsia prowazeki*	*Pediculus humanus*	Humans/squirrels	No	Yes	No	Yes[c]
Tick-borne encephalitis	TBE viruses	*Ixodes* spp.	Ticks	Yes[a]	No	No	Yes[c]
Japanese encephalitis	JE virus	Mosquitoes	Pigs	Yes	No	No[b]	Yes
Murine typhus	*Rickettsia typhi*	*Xenopsylla cheopis*	Rodents	No	Yes	No	Yes
Plague	*Yersinia* pestis	Various fleas	Rodents/squirrels	Yes	Yes	No	Yes
VEE	VEE virus	*Culex* spp.	Rodents	Yes[a]	No	No	Yes
Oropouche	Oropouche virus	Biting midges	Sloths	No	No	No	Yes[c]
Cutaneous leishmaniasis	*L. major/tropica/braziliensis*	Phlebotomine sand flies	Rodents/humans	No	No	No[b]	Yes
Tularemia	*Francisella tularensis*	Ticks/deer flies	Small mammals	Yes[a]	No	No	Yes[c]
African trypanosomiasis	*Trypanosoma brucei gambiense*	*Glossina morsitans*	Humans	No	No	No	Yes[c]
West Nile fever	West Nile virus	Mosquitoes	Birds	No	No	Yes	Yes
African trypanosomiasis	*Trypanosoma brucei rhodesiense*	*Glossina morsitans*	Wild game/cattle	No	No	No	Yes[c]
Lyme disease	*Borrellia burgdorferi*	*Ixodes scapularis/ricinus*	Rodents/birds	No	No	No	Yes
Mayaro fever	Mayaro virus	*Haemogogous* spp.	Primates	No	No	No	No

Sixty percent ($n = 24$) of the top 40 infectious disease threats are primarily transmitted by arthropods, while 4 additional diseases (bacterial and protozoal diarrhea-causing agents, cholera, and Q fever) are occasionally transmitted by arthropods. Only 11 (27.5%) of the top 40 threat agents have no arthropod involvement. [a]Vaccines not approved for general use in the United States. [b]Hand-held diagnostic assay for vector assessment currently under development. [c]Real-time PCR assays for target agent exist; however, these assays may not have been fully validated with arthropod samples.

reduce transmission of the pathogen to deployed military personnel. To effectively reduce transmission it is critical that insecticides or other prevention/control measures be applied in areas where the risk is greatest. The risk of arthropod-borne disease transmission is best expressed by the "entomological inoculation rate" (EIR), which is the number of infectious bites received per person per day in a given area. Key factors affecting the EIR are (i) the number of bites received per person per day and (ii) the proportion of arthropods that are infected and capable of transmitting the pathogen.[22,23] Although it is extremely difficult to determine the proportion of arthropods capable of transmitting a particular pathogen, methods of determining whether an arthropod is infected have improved significantly and can greatly facilitate the estimation of the EIR.

Many arthropod-borne diseases are extremely focal. The distribution of an arthropod-borne disease in a given environment is a reflection of many factors, to include proximity of the pathogen, reservoir and vector in time and space, and the presence of environmental conditions that facilitate rapid development of the pathogen in the vector and allow the vector to survive long enough to transmit the pathogen.[24–30] The mere presence of a potential vector does not in itself indicate that there is a risk of disease. The term "anophelism without malaria" refers to the fact that *Anopheles* mosquitoes capable of transmitting malaria are found in many areas in which malaria transmission is rare or does not occur.[31] For example, malaria-competent *Anopheles* vectors are found in most of North America and Europe yet locally transmitted malaria is essentially nonexistent in these regions.

A number of recent studies suggest that effective prevention and control of vector-borne diseases requires a targeted approach in which maximum resources are committed to areas where the risk of acquiring the particular disease is highest.[28,32–34] For example, Smith et al.[32] found that 20% of people received 80% of all malaria infections in Africa and suggested that targeted control would provide a disproportionate impact and significant benefits, while Vanwambeke et al.[35] felt that dengue control programs in Thailand needed to take into account the temporal and geographic focality of dengue to be truly effective.

Clearly, many factors affect the distribution of vector-borne diseases and the associated risk posed to deployed military forces. Likewise, many different methods can be used to assess potential risk. Presence or absence of a key vector, abundance of the vector, and presence of pathogen-specific antibodies in animal reservoirs or in people living in the area of operations can all provide useful information to help assess

risk and facilitate the development of targeted control programs. With the emergence of field-deployable diagnostic assays, early detection of the pathogen in vector populations has also emerged as an effective method of rapidly assessing risk.[36,37] Ideally, detection of a vector-borne disease threat would occur before the occurrence of cases in deployed military personnel,[37] thereby allowing for the early implementation of vector control measures and minimizing the impact of the disease on military operations.

Methods of Conducting Vector Diagnostics

Vector diagnostics is the detection of disease-causing pathogens within the arthropod vector. One of the earliest methods of determining if an arthropod was infected was to visually examine appropriate organs (e.g., the midgut or salivary glands of *Anopheles* mosquitoes for malaria parasites) under a microscope.[38] This method is normally not appropriate for use during military operations as it is time consuming and requires a high level of training. The development of immunological methods in general, and the enzyme-linked immunosorbent assay (ELISA) in particular, revolutionized the field of vector diagnostics.[39–42] The circumsporozoite protein ELISA for the detection of human malaria parasites in mosquitoes was first developed at the Walter Reed Army Institute of Research (WRAIR) and rapidly became the standard method for assessing mosquito infection rates.[40,41] ELISA assays are capable of testing large numbers of arthropods very rapidly; however, they are not routinely used during military operations because of the amount of equipment required, complexity, and the requirement for a cold chain.

The need for vector surveillance during military deployments led to the development of a series of hand-held immunochromatographic assays. These assays are simple, can be used anywhere, and do not require a cold chain, thereby overcoming most of the challenges associated with the use of ELISA assays. The malaria VecTest assay was developed through a collaborative effort between Navix, Inc. (subsequently Medical Analysis Systems, Inc.) and the WRAIR. The assay detects *Plasmodium falciparum* and *P. vivax* circumsporozoite protein in anopheline mosquitoes and is sold as a kit containing 20 assays, with each assay capable of testing up to 20 mosquitoes in 15 minutes (Fig. 1). Sensitivity ranges from 91 to 100% and specificity from 94 to 99.7%, on the basis of field trials conducted in Africa, Asia, and South America.[43–47] The malaria VecTest kit requires no refrigeration or freezing and is stable up to 24 months at temperatures up to 32°C and for shorter periods at temperatures up to 50°C. The malaria VecTest kit has been assigned a national stock number (NSN: 6550-01-551-5327) and is currently available from VecTOR Test Systems, Inc., Thousand Oaks, CA.

Subsequent to the development of the malaria VecTest kit, the Centers for Disease Control and Prevention (CDC), the WRAIR, and Medical Analysis Systems, Inc. developed a series of five VecTest assay kits for the detection of arthropod-borne viruses. Each kit contains 50 assays, with each

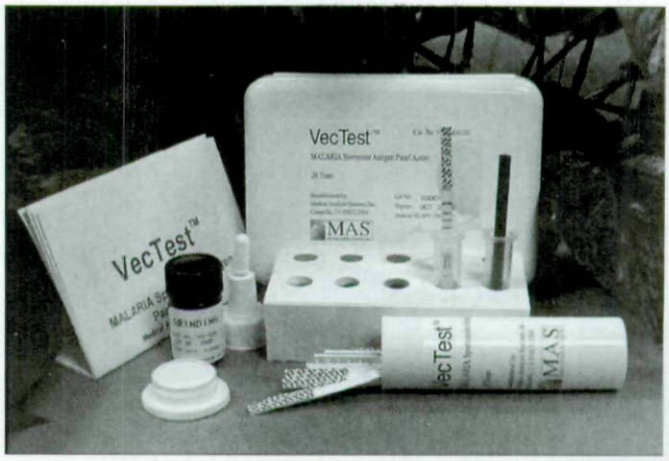

FIGURE 1. The Malaria VecTEST Assay.

assay capable of testing up to 50 mosquitoes. Available kits include (i) a WN virus assay (NSN: 6550-01-533-3943), (ii) a Saint Louis encephalitis (SLE) virus assay, (iii) a combined WN/SLE virus assay, (iv) a combined WN/SLE/EEE virus assay (NSN: 6550-01-533-1564), and (v) a combined WN/SLE/WEE virus assay (NSN: 6550-01-533-1572). Although these assays were originally developed for the detection of viruses in mosquitoes,[47,48] the WN virus assay has also been used to detect WN virus in birds.[49–51] Efforts currently focus on the development of hand-held immunochromatographic assays to detect *Leishmania* parasites and dengue, JE, RVF, SFV, and Ross River viruses.

Although hand-held immunochromatographic assays are ideal for field use, specificity and sensitivity of the assays can be lower than desired. Confirmatory assays, although not absolutely essential, are therefore desirable. Polymerase chain reaction (PCR)-based methods have revolutionized the field of vector diagnostics and can serve as stand-alone screening assays or as confirmatory assays. Initially developed in the 1980s, PCR was first used to detect dengue virus and malaria parasites in mosquitoes in the early 1990s.[52,53] Although traditional PCR equipment has become cheaper, lighter, and easier to use, requirement for gels, multistep procedures, and risk of contamination still preclude routine use under field conditions. However, the development of fluorogenic or real-time PCR assays has overcome many of the limitations of traditional PCR and offers great potential for use during military deployments. Recently, real-time PCR assays were used during Operation Iraqi Freedom to detect *Leishmania* parasites in sand flies, rodents, and human patients.[37,54,55]

The Joint Biological Agent Identification and Diagnostic System (JBAIDS) is the U.S. military's field-deployable platform for real-time PCR assays. JBAIDS is a military-specific version of the Idaho Technology R.A.P.I.D. (Ruggedized Advanced Pathogen Identification Device). The JBAIDS integrates Idaho Technology's LightCycler real-time PCR technology into a portable, impact-resistant package ideal for field use. Distinctive software allows simple "push-button"

use of the JBAIDS by field personnel with minimal training. The JBAIDS is currently used by field-deployable forces such as Army Area Medical Laboratories, Army Combat Support Hospitals, and Navy Forward Deployed Preventive Medicine Units. Although the JBAIDS is primarily being developed for the detection of biological threat agents in clinical samples,[56] it can be used for vector assessment as well. A limited number of assays for the detection of arthropod-borne pathogens are currently available on the JBAIDS platform, to include *Yersinia pestis* (plague), *Francisella tularensis* (tularemia), and *Rickettsia prowazeki* (epidemic typhus); however, to date none of these assays has been validated for use in the detection of pathogens within the arthropod vector.

Rationale for This Study

Until recently, vector diagnostics had not been routinely used during military deployments. However, the development of hand-held assays and real-time PCR assays that can be used in a field environment resulted in the employment of both systems during Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF) by U.S. and German military forces. The goal of this article is to assess the use of these assays during OIF and OEF, to identify issues related to their use, and to make recommendations for future use.

MATERIALS AND METHODS

Collection of Mosquitoes and Sand flies

Mosquitoes and sand flies were collected as part of a systematic effort by U.S. and German preventive medicine (PVNTMED) assets to assess vector populations in areas where military forces were located throughout Iraq and Afghanistan. Standardized guideline on collection procedures were provide to each PVNTMED unit that participated in this study. This effort was initiated in 2003 and has continued to date (2009). Insect collections were primarily made using unbaited CDC miniature light traps; however, German PVNTMED personnel also collected *Anopheles* mosquitoes from inside of tents, buildings, and latrines using a commercially available hand-held mouth aspirator with a hepa filter. Although a variety of individuals from a number of different units were responsible for insect collections, the following procedures were generally used. Light traps were normally placed shortly before sunset and were retrieved soon after dawn. Traps were normally placed within 1 meter of the ground. Fine mesh collecting cups suitable for the collection of sand flies, mosquitoes, and other small insects were used with the light traps. Upon return to the field laboratory, collection cups were placed in a freezer to kill the collected insects. Mosquitoes collected using aspirators were placed in 1-pint screened cartons and returned to the field laboratory where they were killed by freezing at −20°C or using ethanol. Within 1–2 hours the collection cups were removed from the freezer, contents placed in a Petri dish, and mosquitoes and sand flies separated from the remaining insects using a dissecting microscope.

Collections in Iraq and Afghanistan began in 2003 and 2004, respectively, and have continued since then. At the time that this article was prepared, U.S. Army data from 2003–2005 and German data from 2006 were available and were used for all analyses. Sand flies collected in 2003 and 2004 were identified to subfamily (Phlebotominae), while those collected in 2005 were identified to genus (*Phlebotomus* and *Sergentomyia*) and those in 2006 to species. Sand flies were stored frozen at −70°C or in 80–100% ethanol until tested. Mosquitoes were identified to genus,[57] with all anopheline mosquitoes subsequently identified to species using the key of Glick et al.[58] Female anopheline mosquitoes were either tested immediately with the malaria VecTest assay or were stored frozen at −70°C for testing at a later date.

Testing Anopheline Mosquitoes Using the Malaria VecTest Assay

Procedures described in the insert for the malaria VecTest kit were used. All supplies required for running the assay are provided with the kit (Fig. 1). In brief, from 1-10 anopheline mosquitoes were placed into a conical grinding tube and 13 drops of grinding solution dispensed into each tube. A grinding pestle was placed in each tube and rotated vigorously for approximately 1 minute or until the mosquitoes were thoroughly homogenized. A test strip was labeled and placed into the grinding tube containing the mosquito suspension. After 15 minutes the test strip was removed and results read immediately per the insert.

Testing Sand Flies Using Real-Time Polymerase Chain Reaction Assays

DNA Extraction

U.S. units used the Qiagen QIAamp DNA Mini kit to extract DNA from pools of sand flies per procedures described in the product insert. German units homogenized sand flies mechanically using a Roche Diagnostics MagNA Lyser device and extracted DNA using a Roche Diagnostics High Pure PCR Template Preparation kit. Tubes containing extracted DNA were labeled and stored at 4°C or −20°C if PCR was to be performed within 3 days or at −70°C if PCR was to be performed more than 3 days later.

Real-Time PCR Assays

Sand flies were initially tested using a *Leishmania*-genus real-time (fluorogenic) PCR assay modified from an assay developed by Wortmann et al.[59] for testing of clinical samples. The assay was modified so that each reaction contained 1 puReTaq Ready-to-Go PCR bead, 6mM $MgCl_2$, 800 nM of each primer (LEIS L1, LEIS U1), 120 nM of probe (LEIS P1), and 2.0 µl of template DNA. The assay was established and validated at the WRAIR in 2001. Assay validation consisted of an evaluation of the limit of detection of the assay as well as sensitivity and specificity of the assay using cultured *L. major*, *L. donovani*,

L. infantum, and *L. tropica* amastigotes, uninfected sand flies and sand flies infected with *L. major*.[60] The assay was used at the WRAIR for approximately 2 years before deploying the assay to OIF in 2003. A more detailed description of the assay is contained in Coleman et al.[55]

Samples with a mean cycle threshold (Ct) value of 40 were considered negative, while samples with a mean Ct value <40 were considered potentially positive and were retested at least one additional time. Samples testing positive the second time were considered "presumptive positives," while samples testing negative the second time were tested a third time. Samples testing positive on this third test were considered "presumptive positives," while samples testing negative the third time were considered indeterminate (i.e., could not determine if they were true positives or true negatives). An algorithm for the determination of infectivity status is presented in Figure 2.

Four separate laboratories conducted real-time PCR testing, to include the 520th Theater Army Medical Laboratory (TAML), an Air Force biological assessment team (BAT), the WRAIR, and a German military laboratory. The TAML and the BAT were located at Tallil Air Base (TAB), Iraq, while the WRAIR and the German military laboratory were located in Silver Spring, Maryland, and Koblenz, Germany, respectively. Although the same *Leishmania*-genus real-time PCR assay described above was used by each of the four laboratories, the assay was run on different PCR platforms, to include a Smartcycler (Cepheid, Inc.) used by WRAIR, a Lightcycler (Roche, Inc.) used by the TAML and German military laboratory, and a R.A.P.I.D. (Idaho Technology, Inc.) used by the BAT. Although the limited availability of infected sand flies prevented us from fully validating each assay on each platform, whenever possible all positive samples and approximately 10% of negative samples were retested at the WRAIR using the Smartcyler. Although a detailed evaluation of the performance of the different assays at the different sites is beyond the scope of this article, our analysis indicated that the performance of each assay on the different platforms was comparable.[61]

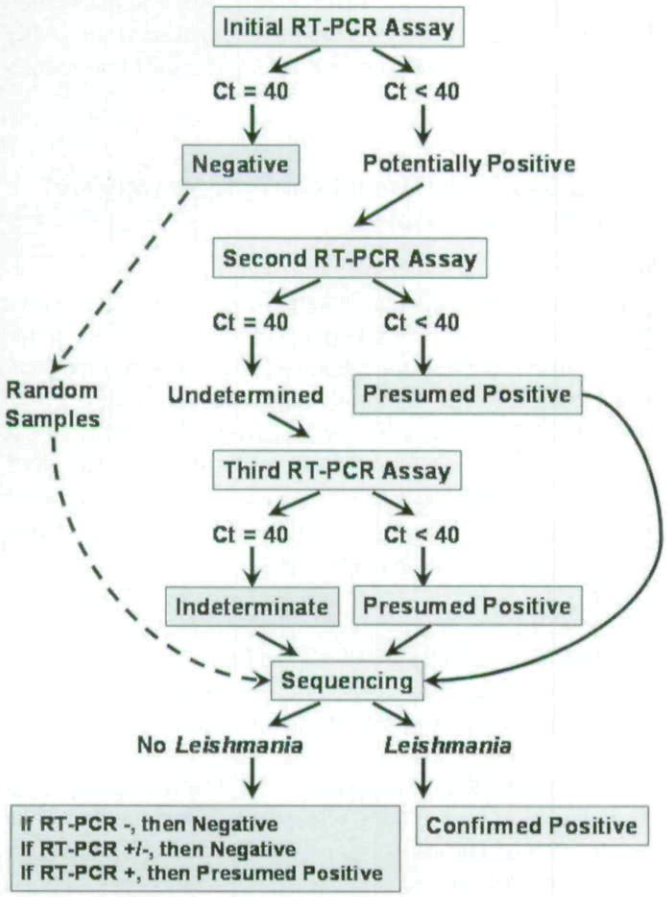

FIGURE 2. Algorithm used to detect and identify *Leishmania* parasites in sand flies collected in Iraq and Afghanistan.

Sequencing of Real-Time PCR Positive Samples

Procedures used for the sequencing of *Leishmania* parasites consisted of (i) a conventional nested PCR reaction, (ii) sequencing of a fragment of the glucose-6-phosphate-isomerase (GPI) gene, and (iii) phylogenetic analysis. Samples determined to be *L. donovani*-complex parasites by GPI sequencing were analyzed further to determine whether they were *L. infantum* or *L. donovani*. An approximately 600-bp region of the "Hyper" gene was used to differentiate between *L. infantum* and *L. donovani*—this region possesses 4 substitution differences and one point deletion/insertion event that differentiate between *L. donovani* and *L. infantum/chagasi* (J.K. Molton, unpublished data).

Standard PCR

A 360-bp fragment of the GPI gene was selected as a target. A first round PCR primer set and a second round nested PCR primer set were selected based on the only *Leishmania* GPI sequence (accession no. X78206) contained in GenBank at the time of the primer design. Each 25-μL reaction contained one puReTaq Ready-to-Go PCR bead, 10 pmol of each primer, and 2 μL of template DNA, either *Leishmania* DNA as purified above or 2 μL of the first round PCR reaction. Cycling conditions included an initial denaturation at 94°C for 2 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 45 seconds, and a final extension at 72°C for 7 minutes. Reactions were performed using an MJ-Research PTC-100 Thermal Cycler. Appropriate negative (water) and positive controls were included in all reaction sets. Positive samples were verified on a 1.5% agarose gel containing ethidium bromide by visualization of a band of the expected size using a transilluminator.

DNA Sequencing

In brief, procedures for sequencing were as follows. The PCR amplification product remaining after gel electrophoresis was purified using the QIAquick PCR purification kit according to the manufacturer's instructions. Automated sequencing was performed using an ABI 3100 genetic analyzer and a

Big-Dye v1.1 or v3.1 sequencing kit according to the manufacturer's instructions. Primer, excess nucleotides, and buffer were removed from the Big-Dye sequencing reaction by eluting the material from a Sephadex G-50 column equilibrated with water. Sequencing of the approximately 600-bp region of the "Hyper" gene was conducted using a BaseStation-100 Automated DNA Sequencer with accompanying BCS and Cartographer software.

Phylogenetic Analysis

Sequences were aligned using the MegAlign program and sequence ends were trimmed to a uniform length. Phylogenetic analyses of aligned sequences were performed using the ClustalW method[62] with a gap penalty of 15 and a gap length of 6.66. The phylogenetic tree generated by MegAlign is a rooted tree with the number of substitution events indicated at the bottom of the tree. Bootstrap replication was used to evaluate the strength of the clustering analysis. Unknown sample sequences were compared to sequences determined for known culture isolates and to other sequences present in GenBank.

RESULTS

Evaluation of Anopheline Mosquitoes for *Plasmodium* Parasites

Iraq

United States PVNTMED units collected a total of 430 anopheline mosquitoes in Iraq. Forty-nine pools containing 191 female *An. pulcherrimus* were tested using the malaria VecTest assay. None of the pools were positive for either *P. falciparum* or *P. vivax* (Table II).

Afghanistan

German PVNTMED personnel collected a total of 1,595 anopheline mosquitoes in Afghanistan, to include 1,030 *An. pulcherrimus*, 348 *An. hyrcanus*, and 217 *An. superpictus* (Table II). A total of 321 pools containing 1,423 female anophelines were tested, of which 7 were positive for *P. falciparum*, 3 for *P. vivax* polymorph 210, and 32 for *P. vivax* polymorph 247. One pool of *An. pulcherrimus* was positive for *P. vivax* polymorphs 210 and 247. Assuming only one mosquito in each pool was infected, the minimum infection rate was 3.2% for *An. pulcherrimus*, 2.8% for *An. hyrcanus*, and 2.0% for *An. superpictus* (Table II).

Evaluation of Phlebotomine Sand Flies for *Leishmania* Parasites

Iraq

United States PVNTMED units collected 148,096 sand flies in Iraq (Table III). A total of 6,633 pools containing 57,696 sand flies were tested using the *Leishmania*-genus real-time PCR assay. Seven hundred twenty-seven pools initially tested positive for *Leishmania* parasites; however, after retesting only 577 were considered true positives, with 150 pools considered indeterminate. Assuming that only one sand fly in

TABLE II. Evaluation of *Anopheles* Mosquitoes Collected During OIF/OEF for Malaria Parasites Using the Malaria VecTest Assay

Country	*Anopheles* Species	No. Collected	No. Tested	No. of Pools	No. Positive	Infection Rate (%)	*Plasmodium* Species[a]
Iraq	*An. pulcherrimus*	430	191	49	0	0.0	N/A
Afghanistan	*An. pulcherrimus*	1,030	942	207	30	3.2	3 Pf, 24 PV-247,[b] 3 PV-210[b]
	An. hyrcanus	348	281	70	8	2.8	8 Pv-247
	An. superpictus	217	200	44	4	2.0	4 Pf

[a]Pf, *Plasmodium falciparum*; Pv, *Plasmodium vivax*. [b]One pool of *An. pulcherrimus* was positive for both PV-210 and PV-247.

TABLE III. Evaluation of Phlebotomine Sand Flies Collected in Iraq and Afghanistan for *Leishmania* Parasites Using a *Leishmania* Genus Real-Time PCR Assay

Country	Year[a,b]	No. of Sand Flies Collected	No Tested	No. of Pools	No. Positive	Infection Rate (%)[c]
Iraq	2003	77,766	30,921	2,816	356	1.15
	2004	46,493	17,713	2,268	208	1.17
	2005	23,837	9,062	1,549	13	0.14
	Total	148,096	57,696	6,633	577	1.00
Afghanistan	2004	1,537	1,014	178	13	1.28
	2005	11,519	1,540	320	22	1.43
	2006	8,442	540	43	8	1.48
	Total	21,498	3,094	541	43	1.39

[a]2006 collections from Afghanistan were made by German Bundeswehr PVNTMED personnel; all other collections were made by U.S. PVNTMED units. [b]Prior to testing, sand flies collected in 2003 and 2004 were sorted to subfamily (Phlebotominae), sand flies collected in 2005 were sorted to genus (*Phlebotomus* and *Sergentomyia*), and sand flies collected in 2006 were sorted to species. [c]Infection rate, number of positive pools divided by the number of sand flies tested (assumes only one sand fly in a pool is infected).

each pool was infected, the minimum field infection rate was 1.00% (Table III). Overall infection rates were almost identical for 2003 (1.15%) and 2004 (1.17%); however, infection rates in 2005 (0.14%) were significantly lower (Pearson's χ^2 test, $p < 0.05$) (Table III). A summary of results for a variety of separate areas in Iraq is presented in Table IV. Over 55% of the sand flies were collected from the vicinity of TAB; however, large (>1,000) numbers were collected from 9 other sites in Iraq. Infection rates ranged from a low of 0% in several areas to a high of 5.6% in Ashraf.

Afghanistan

United States and German PVNTMED units collected a total of 21,498 sand flies in Afghanistan, with 541 pools containing 3,094 sand flies tested for the presence of *Leishmania* parasites using the *Leishmania*-genus real-time PCR assay (Table III). Sixty pools initially tested positive for *Leishmania* parasites; however, after extensive retesting only 43 pools were determined to be true positives, with 17 considered indeterminate (Table III). The minimum field infection rate was 1.39%. Eight PCR-positive pools collected by the German Bundeswehr were positive when subsequently tested using a *L. major*-specific assay. A summary of results for a variety of areas in Afghanistan is presented in Table IV. Large (>1,000) numbers of sand flies were only collected from Kandahar and Mazar-e Sharif. Infection rates ranged from a low of 0% in several areas to a high of 1.7% in Kandahar.

Sequencing of Leishmania

We sequenced a 360-bp region of the GPI gene from 731 pools of sand flies collected in Iraq and Afghanistan by U.S. PVNTMED units (Table V) and 8 samples collected in Afghanistan by the German Bundeswehr. DNA from each of these pools had been previously extracted and assessed using the *Leishmania*-genus real-time PCR assay. These 739 pools included 570 of the 620 PCR-positive samples, 158 of the 167

TABLE IV. Summary of Sand Fly Collections Made in a Variety of Locations in Iraq (2003–2005) and Afghanistan (2004–2006), to Include Real-Time PCR and Sequencing Results

	Sand Fly Collections[a]			Real-Time PCR Assay Results[b]				Sequencing Results[c]					
Location	No. Collected	No. of Traps	X/Trap	No. of Pools	No. Flies Tested	No. Positive	Infection Rate	Ld	Lt	Lm	Ltar	Neg	Total
Iraq													
Tallil Air Base	82,054	1,836	44.7	2,525	26,851	456	1.70	18	3	2[d]	262	238	523
Baghdad	13,523	955	14.2	629	5,471	10	0.18		1		2	16	19
Balad	10,010	1,331	7.5	637	4,754	14	0.29				5	19	24
Tikrit	9,516	1,986	4.8	541	3,094	18	0.58				13	9	22
Diwaniyah	7,993	Unknown	N/A	325	3,262	7	0.21			1	2	18	21
Babylon	5,861	2,082	2.8	562	3,913	8	0.20	1			1	11	13
Muqdadiyah	4,889	Unknown	N/A	231	2,164	7	0.32				5	3	8
Taji	4,488	301	14.9	336	2,538	15	0.59			1	3	17	21
Baquaba	3,029	63	48.1	195	1,583	3	0.19				1	6	7
Al-Asad	2,491	513	4.9	256	1,445	3	0.21				1	6	7
Mosul	975	161	6.1	116	635	3	0.47				2	6	8
Ashraf	685	60	11.4	68	482	27	5.60				12	2	14
Kirkuk	558	42	13.3	51	291	0	0.00						0
Habbaniyah	532	15	35.5	29	254	2	0.79				1	1	2
Al Kut	417	Unknown	N/A	20	298	2	0.67				1	2	3
Ramadi	272	Unknown	N/A	24	240	1	0.42				1		1
Tal Afar	198	598	0.3	34	69	0	0.00						0
Tuz	184	3	61.3	10	86	0	0.00						0
Bayji	174	50	3.5	13	102	1	0.98	1			1		1
Other Sites	247	53	4.7	31	164	0	0.00						0
Total	148,096	Unknown	N/A	6,633	57,696	577	1.00	20	4	3	313	354	694
Afghanistan													
Kandahar	12,112	316	38.3	335	1,888	32	1.69				25	9	34
Mazar-e Sharif	8,442	157	57.8	43	540	8	1.48			8			8
Bagram	263	244	1.1	74	204	0	0.00						0
Salerno	235	50	4.7	46	151	2	1.32				1	1	2
Jalalabad	230	4	57.5	17	153	1	0.65				1		1
Kabul	191	100	1.9	23	140	0	0.00						0
Other Sites	25	2	12.5	3	18	0	0.00						0
Total	21,498	873	24.6	541	3,094	43	1.39	0	0	8	27	10	45

Ld, *L. donovani*-complex; Lt, *L. tropica*, Lm, *L. major*; Ltar, *L. tarentolae*; Neg, samples that did not yield a sequence determined to be *Leishmania*. [a]All collections were made by U.S. military PVNTMED unites except for collections from Mazar-e Sharif, Afghanistan, which were made by German Bundeswehr PVNTMED units. [b]Includes only confirmed PCR-positive samples. [c]A total of 739 samples were sequenced, to include 731 sequenced by the WRAIR, and 8 by the German Bundeswehr (these 739 samples include 570 that were PCR-positive, 158 that were PCR-indeterminate, and 11 that were PCR-negative). [d]These two samples are similar to both *L. major* and *L. tropica* (*L. major/tropica* "like").

TABLE V. Determination of Species of *Leishmania* Parasites Based Upon Sequencing of a Portion of the Glucose 6-Phosphate Isomerase Gene

Sequencing Result	Number (% of Total)	Mean Real-Time PCR Ct Value (STD DEV)
L. donovani complex	20 (2.7)	29.4 (4.82)
L. major[a]	1 (0.1)	31.3 (–)
L. tropica	4 (0.5)	38.0 (1.13)
L. major/tropica "like"	2 (0.3)	28.4 (3.68)
L. tarentolae	340 (46.5)	28.1 (3.74)
Non-*Leishmania* GPI	364 (49.9)	36.6 (2.88)
Total	731 (100.0)	32.7 (5.29)

[a]Eight (8) additional *L. major* sequences were detected by the German Bundeswehr.

PCR-indeterminate samples, and 11 PCR-negative samples. Sequencing indicated that 35 (4.6%) of these 739 samples contained human-pathogenic *Leishmania*, to include 20 samples containing *L. donovani*-complex parasites, 9 containing *L. major*, 4 containing *L. tropica*, and 2 containing a parasite that appeared similar to both *L. major* and *L. tropica* (Table V). Three hundred forty (46.5%) samples contained *L. tarentolae* DNA, while no *Leishmania* DNA was detected in 365 samples (49.8%). A 600-bp region of the "Hyper" gene was sequenced for 14 of the samples that contained *L. donovani*-complex DNA. All 14 samples contained *L. infantum* DNA while none contained *L. donovani* DNA.

Pathogenic *Leishmania* detected from sand flies from Iraq included *L. donovani*-complex parasites, *L. tropica*, and *L. major*. The majority (23/27) of the pathogenic samples from Iraq came from TAB in southern Iraq (Table IV). Eighteen of these samples were *L. donovani*-complex parasites (with 12 of these identified as *L. infantum*), 3 were *L. tropica*, and 2 were similar to both *L. tropica* and *L. major*. Additional pathogenic samples included a *L. tropica*-positive sample from Camp Victory in Baghdad, a single *L. infantum*-positive sample each from Babylon and from Diwaniyah, and a *L. major*-positive sample from Taji (Table IV). Eight *L. major*-positive samples from Mazar-e Sharif were the only pathogenic *Leishmania* detected in sand flies collected in Afghanistan.

Sorting of Sand Flies Before Testing

Pathogenic Old-World *Leishmania* are transmitted by *Phlebotomus* spp. sand flies whereas the nonpathogenic saurian *Leishmania* are transmitted by *Sergentomyia* spp. sand flies.[63] The high proportion of the saurian *L. tarentolae* detected in 2003 and 2004 led to a decision by the WRAIR to separate sand flies by genus in 2005 and to focus on the testing of only *Phlebotomus* spp. sand flies. This was an attempt to minimize the detection of saurian *Leishmania*. In an effort to further refine the testing strategy, German PVNTMED personnel identified all sand flies collected in 2006 to species before testing.

This change in protocol resulted in several immediate observations. First, infection rates in *Sergentomyia* spp. sand flies were five times higher than in *Phlebotomus* spp. sand flies (Table VI). Secondly, *L. tarentolae* was commonly detected in both *Phlebotomus* and *Sergentomyia* spp. sand flies, accounting for 11 of 12 of the *Leishmania* species identified from *Phlebotomus* spp. sand flies and 13 of 13 of those identified from *Sergentomyia* spp. sand flies (Table VI). No pathogenic *Leishmania* were detected in *Sergentomyia* spp. sand flies; however, *L. donovani*-complex parasites were detected in one pool of *Phlebotomus* spp. sand flies. Although the number of samples collected and tested by German PVNTMED personnel was far lower than those tested by U.S. laboratories, the high proportion (8/32) of *P. papatasi* pools infected with *L. major* was noteworthy, as was the 2.5% infection rate.

Relationship Between Real-Time PCR Ct Value and Sequencing Results

Because many samples were positive for *Leishmania* DNA by real-time PCR but were negative when sequenced, we

TABLE VI. Impact That Sorting Sand Flies to Subfamily (Phlebotominae), Genus (*Phlebotomus* or *Sergentomyia*) or Species (*P. papatasi*, *P. caucasicus*, or *S. sintoni*) Had on *Leishmania* Infection Rates

Level Sorted to[a]	No. of Sand Flies	No. of Pools	No. Positive	% Infected	*Leishmania* Species by Sequencing (% of Total)[b]				
					Ld	Lt	Lm	Ltar	Total
2003 and 2004									
Mixed females	49,122	5,232	577	1.17	19 (5.6)	4 (1.2)	3 (0.9)[c]	316 (92.4)	342
Mixed males	575	41	0	0.00					
2005									
Phlebotomus	8,372	1,567	15	0.18	1 (8.3)	0 (0)	0 (0)	11 (91.7)	12
Sergentomyia	2,181	291	20	0.92	0 (0)	0 (0)	0 (0)	13 (100)	13
2006									
P. papatasi	320	32	8	2.5	0 (0)	0 (0)	8 (100)	0 (0)	8
P. caucasicus	40	2	0	0.00					
S. sintoni	180	9	0	0.00					
Total	60,790	7,174	620	1.02	20 (5.3)	4 (1.1)	11 (2.9)	340 (90.7)	375

[a]Sand flies collected in 2003 and 2004 were sorted to subfamily (mixed pools), those collected in 2005 were sorted to genus, and those in 2006 to species. [b]Ld, *L. donovani*-complex; Lt, *L. tropica*; Lm, *L. major*; Ltar, *L. tarentolae*; Neg, samples that did not yield a sequence determined to be *Leishmania*. [c]Includes two samples that were similar to both *L. major* and *L. tropica* (*L. major/tropica* "like").

compared real-time PCR Ct values with sequencing results to determine whether there were any relationships between strength of the PCR reaction and the proportion of samples determined to be *Leishmania* by sequencing (Table VII). As the mean Ct values decreased (i.e., reaction became stronger), the proportion of samples that tested positive for *Leishmania* parasite GPI sequences increased while the proportion of other GPI sequences decreased. For example, 99% of the samples with a mean Ct value <26 matched known *Leishmania* spp. sequences, whereas only 3% of the samples with a mean Ct value between 38 and 39.99 matched any known *Leishmania* spp. sequence (Table VII).

DISCUSSION

Evaluation of the Malaria VecTest Assay

In spite of an intensive surveillance effort, only 430 anopheline mosquitoes were collected in Iraq and all 191 that were tested using the malaria VecTest assay were negative. In contrast, 1,595 anopheline mosquitoes were collected in Afghanistan, with a total of 35 pools infected with *Plasmodium vivax* and 7 with *P. falciparum*. Although we were not able to determine whether any of the infected mosquitoes were capable of transmitting malaria, *An. pulcherrimus*, *An. hrycanus*, and *An. superpictus* are all known vectors of malaria in Southwest Asia.[64,65] These data suggest that malaria rates should be much higher in service members deployed to Afghanistan compared to those deployed to Iraq, and that the majority of cases should be the result of infection with *P. vivax*. Unfortunately, the long incubation period of temperate-strain *P. vivax*,[66] combined with the mobility of U.S. military personnel makes it difficult to determine exactly where vivax malaria infections were acquired.[67] Although 60 soldiers who deployed to Iraq since 2003 have been diagnosed with malaria, only 7 individuals in Iraq during the transmission season had no other documented exposure, suggesting that most of these soldiers acquired their infections elsewhere.[67] In contrast, significant numbers of military personnel appear to have been infected while in Afghanistan.[10] Cimera and Brundage[67] reported that 74 military members with malaria had served in Afghanistan during the transmission season, with 41 (55%) having no other documented exposure risk, while Kotwal et al.[68] reported 38 active duty soldiers from a 725-man Ranger Task Force contracted malaria while operating in eastern Afghanistan in 2002.

Our data suggest that hand-held assays are a remarkably powerful tool with which to assess the threat of vector-borne diseases to deployed military forces. These assays can be used anywhere and can provide real-time feedback. These assays will be most valuable when used to assess the vector-borne disease threat immediately before or soon after moving military forces into a given area—the goal should be the detection of pathogens before the onset of disease in our deployed military forces. Early detection of a pathogen will allow for the implementation of pathogen/vector-specific protective measures that can minimize casualties to our military forces. For example, use of the malaria VecTest assay in Afghanistan clearly demonstrated that infected anopheline mosquitoes were present—these positive assay results were extremely useful in obtaining command support for mandatory use of PPM. In contrast, all of the mosquitoes tested with this assay at TAB, Iraq were negative, suggesting that malaria was not present or was exceedingly rare at this site. Information on the scarcity of anopheline mosquitoes in most areas in Iraq combined with the negative malaria VecTest assay results helped medical authorities implement a policy in which mandatory malaria prophylaxis was discontinued. We believe that hand-held vector assays are a valuable force multiplier and should play a key role in the Deployment Environmental Surveillance Program directed by the U.S. Army Center for Health Promotion and Preventive Medicine (USACHPPM).

TABLE VII. Relationship Between Threshold Cycle (Ct) Values of a Real-Time *Leishmania* Genus PCR Assay and the Proportion of Samples Determined to Be Positive Upon Retesting With the Same PCR Assay and by Sequencing of a Portion of the Glucose-6-Phosphate-Isomerase Gene

Real-Time PCR Assay Results					Sequencing Results		
Initial Ct Value[a]	No. of Samples	No. (%) Negative[b]	No. (%) Positive[b]	No. (%) Indeterminate[b]	No. Sequenced	No. (%) *Leishmania*	No. (%) Other
40	6,352	6,352 (100)	0 (0)	0 (0)	11	0 (0)	11 (100)
38–39.99	147	0 (0)	7 (5)	140 (95)	131	4 (3)	127 (97)
36–37.99	113	0 (0)	88 (78)	25 (22)	110	8 (7)	102 (93)
34–35.99	83	0 (0)	82 (99)	1 (1)	73	20 (27)	53 (73)
32–33.99	72	0 (0)	71 (99)	1 (1)	68	32 (47)	36 (53)
30–31.99	89	0 (0)	89 (100)	0 (0)	86	61 (71)	25 (29)
28–29.99	78	0 (0)	78 (100)	0 (0)	71	63 (89)	8 (11)
26–27.99	71	0 (0)	71 (100)	0 (0)	62	61 (98)	1 (2)
<26	126	0 (0)	126 (100)	0 (0)	119	118 (99)	1 (1)
Total	7,131	6,352 (89)	612 (9)	167 (2)	731	367 (50)	364 (50)

[a]The Ct value is the number of cycles at which the sample was considered positive. The lower the value the stronger the reaction. A Ct value of 40 is considered negative. [b]The determination as to whether the sample was considered positive, negative, or indeterminate was based on retesting of the sample as outlined in Figure 2.

Hand-held vector assays should also be integrated into PVNTMED units of all services.

The malaria VectorTest (trademark of VecTOR Test Systems, Inc.) assay and the various other VecTest (trademark of products from Medical Analysis System, Inc., now a part of ThermoFisher) assays for the detection of EEE, WEE, SLE, and WN viruses are currently the only hand-held assays available for vector surveillance during military deployments. A variety of additional VectorTest assays, to include assays for the detection of *Leishmania* parasites and dengue, RVF, JE, SFV, and Ross River viruses, are currently being developed and could potentially be fielded within the next several years. The ultimate goal is the fielding of hand-held vector assays for all of the top threat agents identified in Table I.

Evaluation of Real-Time PCR Assays for Leishmania *Surveillance*

In contrast to hand-held vector assays that can be used almost anywhere,[45] real-time PCR assays normally require a power supply and a cold chain. In spite of these limitations, real-time PCR assays can be used under a variety of field conditions, to include tents and other portable structures. Although not as rapid as the hand-held assays, under optimal conditions DNA can be extracted and assay results obtained within several hours. We found that the *Leishmania*-genus real-time PCR assay was a useful tool that allowed us to screen hundreds of sand flies each day for *Leishmania* parasites. However, as with all other tools, the limitations of the assay must be understood when interpreting results.

The high infection rates in sand flies collected in the vicinity of TAB and several other areas in Iraq suggested that service members stationed in these areas were at high risk of becoming infected with *Leishmania* (Table IV). It was only once sequencing of the 360-bp region of the GPI gene had been completed that we were able to determine that the actual threat was much lower than originally suggested, since the nonpathogenic *L. tarentolae* accounted for the majority of the PCR-positive samples. Before our deployment to Iraq in 2003, there was little information available on the abundance of nonpathogenic *Leishmania* in sand flies in the Middle East. Although we realized that nonpathogenic species such as *L. tarentolae*, *L. turanica*, and *L. gerbilli* were potentially present in Iraq, there was no published data that suggested nonpathogenic *Leishmania* were present or that they would account for over 90% of the PCR-positive samples from sand flies. Unfortunately, the *Leishmania*-genus real-time PCR assay is not capable of differentiating pathogenic from nonpathogenic species.

Clearly, appropriate targets must be developed for assays that will be used in vector surveillance. Although a *Leishmania*-genus real-time PCR assay is acceptable for use with human samples, as any *Leishmania* detected in a human sample is intuitively pathogenic, our data demonstrate that a *Leishmania*-genus assay cannot be used independently to assess the medical threat posed by sand flies. Sequencing of appropriate targets (e.g., the GPI and "Hyper" genes as done in this study) can be used to identify the *Leishmania* species; however, sequencing is currently not possible in a field environment and can take several weeks. Assays that can differentiate pathogenic from nonpathogenic *Leishmania* are clearly needed, as are species-specific assays. We have developed two assays specific for *L. donovani*-complex parasites and one for *L. major* and are currently developing assays for *L. tropica* and *L. tarentolae*. We have also developed an assay that is specific for pathogenic *Leishmania* parasites of the Old World, to include *L. tropica*, *L. major*, *L. aethiopica*, and *L. donovani*-complex parasites. This assay does not detect nonpathogenic species. Once fully validated, these assays will allow for a more rapid assessment of the medical threat posed by sand flies in the Middle East.

In addition to requiring additional species-specific assays, it is critical that the performance of each assay be well established before use in an operational setting. Procedures used to validate initial test results should be established, as should a rubric for identifying the causative agent. At the onset of OIF, we had completed initial validation of the *Leishmania*-genus assay using laboratory-infected sand flies and had calculated the limit of detection for *L. major*. We had also developed assays specific for *L. major*, *L. tropica*, and *L. donovani*-complex parasites; however, we had not yet fully validated these assays. Although we knew that these species-specific assays were approximately 10 times less sensitive than the *Leishmania*-genus assay, we had not determined the limit of detection for each assay nor had we evaluated cross-reactivity of the assays. Initially, all samples that tested positive with the *Leishmania*-genus assay were subsequently tested using the *L. major*, *L. tropica*, and *L. donovani*-complex assays. Over 90% of the samples testing positive using the *Leishmania*-genus assay were negative with the species-specific assays. Because of our incomplete understanding of the performance of each assay, we were not able to determine whether the species-specific assays were negative because (i) they were less sensitive than the *Leishmania*-genus assay, (ii) the *Leishmania*-genus assay was yielding false positive results, or (iii) the *Leishmania*-genus assay was detecting a species of parasite not recognized by the species-specific assays. The development of a testing algorithm (Fig. 2) that included multiple retesting using real-time PCR and sequencing of a portion of the GPI gene was an attempt to overcome these limitations.

When using real-time PCR assays it is also important to establish realistic cut-off values that can be used to determine whether samples are positive, negative, or indeterminate. When establishing the *Leishmania*-genus PCR assay at WRAIR, our laboratory data suggested that all Ct values <40 should be considered positive. However, when our field data were carefully analyzed (Table VII) to include the use of sequencing in our testing paradigm, it became clear that it was necessary to establish an indeterminate range (i.e., not possible to determine if the result was a true positive or a true negative). Retesting of samples that initially tested positive

with the real-time PCR assay suggested that an appropriate cut-off might be at a Ct of 38, as only 5% of samples with a Ct between 38 and 39.99 were positive upon retesting, whereas 78% of samples with a Ct between 36 and 37.99 tested positive upon retesting (Table VII). Although sequencing proved to be a useful tool for confirming the identify of parasites, it was difficult to determine if a negative sequencing result meant that the original PCR assay result was a false positive or whether the large number of PCR-positive samples that were negative by sequencing reflected limitations of our sequencing procedures. Our results clearly indicate that confirmatory assays are a key component of any testing program. Ideally, any confirmatory assay should have a limit of detection similar to or below that of the screening assay and should target a separate genomic region of the target pathogen.

In addition to refining our test procedures as a means of conclusively identifying *Leishmania* parasites in sand flies, we also attempted to determine whether sorting sand flies to genus and/or species could eliminate the nonpathogenic *Leishmania*. Since *Sergentomyia* spp. sand flies are believed to be the vectors of *Sauroleishmania* (e.g., *L. tarentolae*),[63] we hoped that by focusing testing on *Phlebotomus* spp. sand flies we would reduce or eliminate positive samples resulting from detection of nonpathogenic species of *Leishmania*. Although *Leishmania* parasites were detected in *Sergentomyia* spp. sand flies much more frequently than in the *Phlebotomus* spp. sand flies, *L. tarentolae* nevertheless accounted for 92% (11/12) of the *Leishmania* samples detected in *Phlebotomus* spp. sand flies. We were not able to determine whether the *Phlebotomus* spp. sand flies were capable of transmitting *L. tarentolae*; however, these data clearly demonstrated that sorting of sand flies to genus will not eliminate the detection of nonpathogenic *Leishmania*.

To date, only three military personnel deployed to Iraq since 2003 have developed visceral leishmaniasis. However, our data demonstrate that parasites that cause visceral leishmaniasis posed a threat to military personnel in Iraq in 2003 and 2004. The fact that more symptomatic cases have not yet occurred in deployed military personnel is not unexpected as visceral leishmaniasis caused by *L. infantum* has historically been considered a disease of young children who are malnourished and/or immunocompromised.[69] Among residents in the Mediterranean basin, symptomatic visceral leishmaniasis in adults is almost exclusively a result of concomitant infection with *L. infantum* and human immunodeficiency virus.[69,70] The fact that deployed military personnel are presumably healthy, well-fed, immune-competent adults suggests that the risk of developing symptomatic visceral leishmaniasis resulting from infection with *L. infantum* is low; however, there may be potential long-term concerns involving the disease. *Leishmania* parasites can persist in the body for life, even following successful treatment of symptomatic individuals,[71] and asymptomatic carriers can become symptomatic following suppression of their immune system.[70] Although the exact number of military personnel actually exposed to parasites that cause visceral leishmaniasis may never be determined, as there are currently no FDA-licensed tests that can be used to assess exposure, our data clearly demonstrate that sand flies infected with *L. infantum* were present in areas where U.S. military personnel were stationed.

Concept of Operations for Vector Diagnostics

Vector diagnostics provides deployed military forces with a powerful tool to assess the threat from vector-borne diseases. Vector diagnostics is not a stand-alone process, but rather is part of an integrated prevention effort consisting of (i) vector surveillance, (ii) vector identification, (iii) vector diagnostics, (iv) individual protective measures, and (v) collective protective measures. When this integrated prevention process is effectively implemented it can result in a rapid assessment of the vector-borne disease threat and assist in the establishment of disease prevention programs, such as the "Leishmaniasis Control Program" established at TAB, Iraq in 2003.[37] The development of diagnostic procedures for identifying pathogens in arthropods has lagged far behind the other four steps in this process. However, technological advances since the early 1990s have led to the creation of diagnostic tools that allow trained personnel to identify pathogens in arthropods in a rapid and efficient manner. The following is our proposed concept of operations (CONOPS) for the use of vector diagnostics:

Assays Used

We propose a two-tiered system for use by deployed military forces, with more extensive capabilities available in fixed medical facilities such as the WRAIR or USACHPPM. In this two-tiered system, hand-held immunochromatographic assays are used as screening tools and real-time PCR assays are used as confirmatory tools. The hand-held assays will ideally target a broad range of pathogens (e.g., genus-level assays), while each real-time PCR assay would ideally target a single pathogen (e.g., species-level assays). For example, a *Leishmania*-genus hand-held screening assay could be complemented by real-time PCR assays specific for *L. major*, *L. tropica*, and *L. donovani*-complex parasites. Confirmatory assays should consist of two real-time PCR assays that recognize two separate targets on different genes for each pathogen of interest. Currently, very few hand-held or real-time PCR assays are available for vector diagnostics (Table I). The development and validation of additional assays and employment practices should be a priority of the Military Infectious Disease Research Program administered by the U.S. Army Medical Research and Material Command.

Fixed facilities such as the WRAIR and the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) possess a variety of more sophisticated diagnostic capabilities that would not normally be available to deployed forces, to include procedures such as sequencing of genomic material and culturing and subsequent identification of pathogenic agents. These fixed facilities are a valuable

resource that can facilitate the identification of vector-borne pathogens.

The Armed Forces Pest Management Board (www.afpmb.org) maintains a database that lists a variety of vector assays that have been developed by various military laboratories and the CDC. The intent of this spreadsheet is to provide potential users with a list of assays that may be used to support their individual requirements. Inclusion of a specific assay in this spreadsheet does not imply that the assay has been endorsed by the AFPMB except where noted.

Assay Validation

If possible, all assays used during military deployments should be fully validated for all target organisms. Standard performance criteria should be calculated for each assay, to include limit-of-detection, sensitivity, specificity, and positive- and negative-predictive value. Ideally, performance should be evaluated using infected arthropods. Unfortunately, the challenges associated with obtaining infected arthropods will frequently preclude comprehensive evaluation of assay performance. In these instances, it is critical that the limitations of the assay be fully understood and that these limitations be considered when interpreting assay results.

Units Using Vector Diagnostics

Hand-held assays are primarily used by units actually conducting vector surveillance operations, such as the PVNTMED Section of a Brigade Combat Team or an Army PVNTMED Detachment. These units will normally not be equipped with real-time PCR assays and will therefore need to ship specimens to a medical laboratory for confirmatory testing. Real-time PCR assays are primarily used by units with sophisticated laboratory capabilities beyond those found in a Brigade Combat Team or Army PVNTMED Detachment. Units with real-time PCR capabilities include Army Area Medical Laboratories, Navy Forward Deployed PVNTMED Units, and Air Force Biological Assessment Teams, among others. Although these sophisticated laboratories may on occasion conduct vector surveillance operations, their primary mission is testing environmental samples to assess the medical threat to deployed forces. Therefore, in most instances samples will be shipped to these laboratories by the units actually conducting vector surveillance operations.

Collection of Arthropods

A variety of procedures may be used to collect arthropods that will subsequently be tested for the presence of pathogens. The collection procedure should not interfere with the diagnostic assay to be performed. Although the majority of collection procedures will not interfere with diagnostic procedures, some procedures (e.g., sticky traps) could potentially interfere and should not be used unless proven to be compatible with diagnostic procedures. Standardized sampling procedures should be used whenever possible, with guidance on procedures provided before the onset of sampling. The type of trap used, height of trap, period of trap placement, and numbers and locations of traps should all be standardized, as should the use of attractants such as dry ice or compressed carbon dioxide. Data collection begins at this step and the collectors must enter and maintain the data in electronic or written form so that this information can be forwarded with and linked to the samples. Basic data should include location, date/time, collector's name, collection method, and habitat information. Other parameters can be added to the data set as needed by the study design.

Killing, Preserving, and Storing Arthropods

The procedure used to kill, preserve, and store collected arthropods should not interfere with the diagnostic assay that will subsequently be performed. Acceptable methods of killing arthropods include freezing or immersion in ethanol. While a variety of additional methods (e.g., heat or use of chemicals such as ethyl acetate or potassium cyanide) are frequently used to kill collected arthropods, further studies are needed to ensure that these procedures are compatible with each diagnostic procedure to be used. Each diagnostic assay should provide detailed information on how specimens should be stored before testing. In general, specimens should be tested immediately or stored frozen at −20°C or colder (−70°C is preferable if samples will be stored for months or years before testing) or in 90–100% ethanol. A variety of additional storage methods may be compatible with test procedures; however, these storage procedures should not be used unless empirical data demonstrate that they will not adversely affect the performance of the diagnostic procedure. Unnecessary freeze–thaw cycles should be minimized as this can affect assay results.

Pooling Specimens

In an unconstrained environment, each arthropod collected would be tested individually; however, because of cost and time this is rarely feasible. Instead, groups of "like" specimens are normally combined into pools of 5–50 individuals and the pools tested. Whenever possible, arthropods in a single pool should come from a single collection made at a particular site and at a particular time. For example, arthropods in a given pool would come from a single light trap collection made on a single night. In many cases, the specimens are placed into pools during the sorting process to facilitate future specimen testing.

Labeling Samples

Each sample (e.g., vial containing whole arthropods, homogenized arthropods, or DNA/RNA extracts) should receive a unique identifier that allows that particular sample to be linked to the collection data. Identifiers should be written directly on vials using a permanent, water/ethanol resistant marker or using preprinted labels intended for use with cryovials.

Homogenization/Extraction Procedures

A variety of procedures may be used for the homogenization of samples and/or the extraction of DNA or RNA. Arthropods

tested using hand-held VecTest assays are homogenized in a grinding buffer using a small mortar and pestle provided with the kit, with the dipstick placed directly into the homogenate. Samples to be tested using real-time PCR assays are homogenized and then DNA or RNA are extracted using a variety of different protocols. Safety issues related to homogenization procedures are discussed further in the biological safety section.

Assay Protocols

Each assay used for vector diagnostics should include a detailed protocol specifying procedures to be used and limitations of the assay. A point-of-contact (telephone number and e-mail address) should be provided so that questions regarding the assay can be rapidly addressed. Whenever possible, a given surveillance program should be directed by a central coordinating agency (e.g., USACHPPM) so that protocols can be implemented consistently and data from different sites compared to one another.

Use of Multiple Test Procedures

Although most samples will be tested using only a single procedure, in some instances additional diagnostic testing may be required. Reasons for conducting additional testing include confirmation of the identity of a positive sample or testing for additional pathogens. In all instances where multiple test procedures may be used, a protocol should be established that will allow for subsequent testing of samples. For example, samples that are stored in ethanol cannot subsequently be used to culture viable pathogens, while the grinding buffer used in certain assays may be incompatible with other test procedures.

Interpretation of Results

All test results, whether positive or negative, should be recorded. Negative test results have limited value, particularly when a small number of samples have been evaluated. Negative results do not indicate that the specific pathogen is not present, but rather that the pathogen was not detected in the sample population. Although a comprehensive surveillance program would ideally collect periodic samples from a variety of sites over the course of a transmission season, in many instances samples are collected from a limited number of sites over a short period of time. In these instances negative test results may be the result of surveillance that misses key sites or surveillance conducted at a time when infected arthropods were not present. Although the value of negative test results increase as the sample size increases, it is difficult to estimate a minimum number of negative samples required to assure a high degree of confidence that a particular pathogen has a low or nonexistent threat because many factors affect the efficiency of pathogen transmission.

Positive samples are much more valuable than negative samples, as they indicate that a particular pathogen was present at a certain location on a certain date. Positive samples can be used to calculate minimum field infection rates that can provide a rough estimate of the magnitude of the threat. However, as described in this article, care must be used when interpreting positive assay results, as the organism detected may not be pathogenic to humans, the vector may not be infectious, or the vector may not feed on humans.

Reporting of Test Results

Standardized forms should be used for recording and reporting diagnostic assay results. These forms will ideally be prepared by a single organization such as the USACHPPM and reviewed and approved by the AFPMB. Copies of all data and reports should be provided to the organization that collected the samples and to a single organization that will archive the data. Summaries of data should be provided to all organizations with an inherent interest in vector diagnostic test results, to include the National Center for Medical Intelligence (formerly the Armed Forces Medical Intelligence Center), the AFPMB, the USACHPPM, and through the medical chain-of-command to the Combatant Commands Surgeon's office.

Biological Safety Considerations

The use of vector diagnostics poses a potential risk to personnel conducting the testing. The primary risk is exposure to aerosolized pathogens during homogenization of the arthropods. Although to our knowledge no disease transmission has ever occurred during processing of field-collected arthropods for vector diagnostics, the CDC publication "Biosafety in microbiological and biomedical laboratories" has documented 151 instances in which a laboratory worker was infected with an arthropod-borne virus—the majority of these infections are believed to have resulted from aerosolization of the virus.[72] Although no specific guidelines for the handling of field-collected arthropods are available, the CDC recommends that Biosafety Level 2 practices be used for processing field-collected mosquito pools for West Nile virus.[73] In the absence of specific Department of Defense (DoD) or Department of Health and Human Services (HHS) guidelines on the handling of field-collected arthropods, whenever possible arthropods should be homogenized in a sealed tube containing a grinding medium or diluent that will inactivate arboviruses and other easily aerosolized pathogens.[74] Unfortunately, the use of diluents that inactivate pathogens may preclude the use of these samples with some diagnostic assays.

Transport of Specimens

The U.S. Code of Federal Regulations (CFR) Title 49 is the document that governs the transportation of hazardous materials, to include infectious substances. Detailed information on the shipment of diagnostic specimens can be found in the USACHPPM fact sheet "Packaging hospital samples and specimens for transport" (http://chppm-www.apgea.army.mil/hmwp/Factsheets/Transport Summary.htm). Arthropods, to include homogenized samples and/or DNA/RNA extracts, are considered general diagnostic specimens unless a specific pathogen has been identified or is suspected. Regulations

pertaining to the air shipment of general diagnostic specimens include 9 CFR (Animal and Plant Health Inspection Service), 21 CFR (Food and Drug Administration), and 42 CFR (Centers for Disease Control and Prevention). To ensure that proper protection is in place to contain any undetected pathogenic micro-organisms, general diagnostic specimens should be packaged in accordance with International Air Transportation Association (IATA) Dangerous Goods regulations. In general, procedures for packing general diagnostic specimens for air shipment include (i) sample placed in a primary watertight receptacle with a leakproof seal, (ii) watertight receptacle wrapped in absorbent material, (iii) wrapped container placed in a secondary watertight receptacle, (iv) the entire package is placed in a strong outer packaging approved by the U.S. Department of Transportation (DOT) for transport of the hazardous material, (v) itemized list describing contents placed in the unsealed shipping box, and (vi) designated certifying official approves package for transport. If needed, ice, dry ice, or prefrozen packs should be placed between the secondary watertight receptacle and the outer packaging. Packages containing dry ice must permit the release of carbon dioxide.

Diagnostic samples in which an infectious agent has been detected are no longer considered general diagnostic samples but rather are considered infectious substances. Infectious substances are defined as "a viable micro-organism, or its toxin, that causes or may cause disease in humans or animals, and includes those agents listed in 42 CFR 72.3 of the regulations of the Department of Health and Human Services or any other agent that causes or may cause severe, disabling or fatal disease." Very stringent regulations pertain to the shipment of infectious substances, with requirements described in detail in 49 CFR Part 173.196. The shipment of infectious substances requires coordinated action by the shipper, the transporter, and the receiver to ensure safe transport and arrival on time. Based on the definition of infectious substances as a "viable micro-organism," diagnostic samples containing a pathogen inactivated using appropriate grinding media or diluent are considered general diagnostic samples and not infectious substances. As such, the less stringent procedures pertaining to the shipment of general diagnostic samples apply. Depending on the type of samples (e.g., dead arthropods, general diagnostic sample, or infectious substances), appropriate CDC or U.S. Department of Agriculture (USDA) permits may be required to ship samples to the United States.

Biological Select Agents and Toxins

Certain microbial pathogens and toxins that potentially pose a severe threat to human, animal, or plant health are referred to as biological select agents and toxins (BSATs). The U.S. Department of Health and Human Services and the USDA have regulatory authority over BSAT that can affect human and animal/plant health, respectively. A current list of USDA and HHS regulated BSATs can be found at http://www.aphis.usda.gov/programs/ag_selectagent/ag_bioterr_toxinslist.html and http://www.selectagents.gov/, respectively. Laws regulating BSATs include Section 511 of the *Antiterrorism and Effective Death Penalty Act of 1996,* the *Uniting and Strengthening America by Providing Appropriate Tools Required to Intercept and Obstruct Terrorism Act of 2001* (USA PATRIOT Act), and the *Public Health Security and Bioterrorism Preparedness and Response Act of 2002* (Bioterrorism Act). On March 18, 2005, HHS and USDA published the final Select Agent Regulations (42 CFR Part 73, 7 CFR Part 331, 9 CFR Part 121) in the *Federal Register.* Subsequent to the publication of these regulations, the DoD and each of the military services have published specific instructions pertaining to BSATs, to include DoD Instructions 5210.88 (Safeguarding Biological Select Agents and Toxins) and 5210.89 (Minimum Security Standards for Safeguarding Biological Select Agents and Toxins), Army Regulations 50-1 (Biological Surety), and 190-17 (Biological Select Agent and Toxins Security Program), and Air Force Policy Directive 10-39 (Safeguarding Biological Select Agents and Toxins).

A number of BSATs are transmitted by arthropods, to include African swine fever, bluetongue, CCHF, EEE, JE, RVF, TBE, and VEE viruses, as well as *Rickettsia prowazekii, R. rickettsii, Francisella tularensis,* and *Yersinia pestis.* Each of these pathogens could potentially be detected in arthropods during military deployments. Because of the severe threat posed by BSATs, the handling, transport, security, destruction, and reporting of these agents are highly regulated. While the various regulations provide clear guidance on procedures used within the United States, there is little guidance on the detection and identification of BSATs in vectors during military deployments. Accepted practice during military deployments is to implement procedures that best meet the intent of relevant U.S. BSAT laws and regulations.

A key issue during military deployments is to determine whether a diagnostic sample should be considered a BSAT, as determination that a sample is a BSAT triggers a variety of specific actions/responses. In general, diagnostic samples are only considered to contain BSATs once a viable pathogen has been identified using a confirmatory assay (42 CFR 73). As described previously, a true confirmatory assay should consist of two separate, validated tests that detect different targets on different genes. There are currently no field-deployable vector assays that meet this requirement; therefore, vector samples are normally considered "presumptive positives" and would not be considered BSATs. However, the future development and fielding of true confirmatory assays could potentially result in vector samples being classified as BSAT. An additional issue is the fact that procedures used to prepare samples for both screening and confirmatory diagnostic assays frequently inactivate any pathogens that are present, so that even though a confirmatory assay identified a particular pathogen, that sample would not be considered BSAT as no viable pathogen is present. However, laboratory personnel should use caution when making a determination that a positive sample is not a BSAT, as it is extremely difficult in a field setting to determine whether a viable pathogen is

present. Additionally, any portion of the diagnostic sample that did not undergo nucleic acid extraction or other sterilizing procedures that would have inactivated the infectious virus may still contain viable BSATs (if identified by a confirmatory assay) and should be destroyed within 7 days as specified in 42 CFR 73. To further complicate matters, nucleic acid from positive-stranded RNA viruses can be used to produce infectious virus—this material would be considered a BSAT if detected using a confirmatory assay.

Clearly, personnel conducting diagnostic testing for BSATs should understand the rules and regulations pertaining to BSATs. Criteria for determining whether a sample contains a BSAT should be established, as should procedures for securing, transporting, and destroying these samples per Army, DoD, and U.S. laws and regulations. Personnel conducting diagnostic testing must also understand that samples that do not meet the strict definition of a BSAT may still pose a considerable threat to anyone exposed to these samples. For example, a sample that tests positive for RVF virus using a hand-held screening assay is not considered a BSAT; however, a potentially lethal virus may still be present in the sample if the grinding diluent does not completely inactivate the pathogen.

SUMMARY

Vector diagnostics are a powerful tool that can greatly facilitate the assessment of the threat posed by arthropod-borne diseases. In the absence of appropriate vaccines or prophylactic drugs, appropriately used vector diagnostics can provide an early warning system that can be used to mitigate the threat of vector-borne diseases. The goal of the diagnostics effort described in this article was to provide the various PVNTMED units stationed in Iraq and Afghanistan with information on the vector-borne disease threat in their respective areas of operation. These PVNTMED units were carrying out vector control operations on a routine basis, and we believed that the rapid evaluation of mosquitoes and sand flies for the presence of *Plasmodium* and *Leishmania* parasites, respectively, and the dissemination of results would allow the PVNTMED units to more effectively develop control programs that focused on areas that were at greatest risk for disease transmission. During OIF, implementation of a vector diagnostics program during the weeks following the invasion of Iraq resulted in the determination that leishmaniasis posed a significant threat to coalition military forces.[37] This determination was made before the detection of any human cases and resulted in the aggressive implementation of prevention and control measures. Unfortunately, the diagnostic assays that were used did not adequately differentiate pathogenic from nonpathogenic *Leishmania*, resulting in an overestimate of the risk posed to deployed military forces. Nevertheless, we believe that the sand fly surveillance program provided valuable information that raised the awareness of the leishmaniasis threat to deployed military forces in both Iraq and Afghanistan. Currently, the USACHPPM continues to test sand flies collected in Iraq and Afghanistan for *Leishmania* parasites, and the WRAIR and other organization such as the Air Force Institute for Operational Health continue to develop improved diagnostic assays for vector surveillance.

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